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Autoimmune Thrombocytopenic Purpura (AITP) and Acquired Thrombasthenia Due to Autoantibodies to GP IIb-IIIa in a Patient With an Unusual Platelet Membrane Glycoprotein Composition

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The subject (E.B.) is a 63-year-old woman with autoimmune thrombocytopenic purpura (AITP) who was first examined some 6 years ago with symptoms of epistaxis and gum bleeding, severe thrombocytopenia, and large platelets. Her serum tested positively with control platelets in the MAIPA assay performed using monoclonal antibodies (MoAb) to glycoprotein (GP) IIIa (XIIF9, Y2/51), yet was negative in the presence of MoAbs to GP IIb (SZ 22) or to the GP IIb-IIIa complex (AP2, P2). The patient's platelets failed to aggregate with all agonists tested except for ristocetin. IgG isolated from the patient's serum inhibited ADP-induced aggregation of control platelets. Unexpectedly, flow cytometry showed an altered expression of membrane glycoproteins on the patient's platelets. Levels of GP Ib-IX were much higher than previously located by us in platelets. In contrast, the expression of GP IIb-IIIa was about half that seen with control subjects. When Western blotting was performed, a striking finding was a strong band of 250 kDa recognized by a series of MoAbs to GP Ibα in addition to the band in the normal position of GP Ibα. Finally, ADP-stimulated (E.B.) platelets failed to express activation-dependent epitopes on GP IIb-IIIa as recognized by PAC-1, AP6, or F26 and additionally gave a reduced P-selectin expression after thrombin addition. In conclusion, we present a novel patient with a severely perturbed platelet function where an altered membrane GP profile is associated with the presence of an autoantibody recognizing a complex-dependent determinant on GP IIb-IIIa and inhibitory of platelet aggregation. Am. J. Hematol. 57:164–175, 1998.

Key words: platelets; GP IIb-IIIa complexes; autoantibodies; autoimmune thrombocytopenic purpura

INTRODUCTION

Autoimmune thrombocytopenic purpura (AITP) is an autoimmune disease caused by circulating antibodies that react with target antigens on the platelet membrane. In recent years, the development of highly specific and sensitive tests for characterizing serum or platelet-bound antibodies such as MAIPA (''Monoclonal Antibody Immobilization of Platelet Antigen'') [1], capture assays using immunobeads [2], MACE (''Modified Antigen Capture ELISA'') [3], and classic immunoprecipitation and immunoblotting procedures [4,5] has resulted in the demonstration that glycoprotein (GP) IIb-IIIa and GP Ib-IX complexes bear the majority of the antigenic targets recognized by antiplatelet antibodies in AITP patients [1–9]. Nevertheless, isolated cases of autoimmune thrombocytopenia have been reported to be caused by antibodies directed to other glycoproteins such as GP Ia-IIa, GP IV, and GP V [10–12].

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Studies on AITP patients have suggested that antiplatelet antibodies that affect platelet function may not be uncommon [13–17]. Nevertheless, the number of reports on well-characterized functionally active antiplatelet antibodies remains limited. This may be due to the fact that in most cases the thrombocytopenia is considered sufficient to explain the bleeding diathesis and further studies are not performed. Indeed, antibody-mediated loss of platelet function has most often been described in patients where the presence of antibody specific for GP IIb-IIIa complexes is not accompanied by thrombocytopenia, or where improvement in the platelet count during therapy is not paralleled by a reduced bleeding tendency.

Glanzmann's thrombasthenia (GT) is an autosomal recessive disorder resulting in a moderate to severe bleeding tendency from early life [18]. The underlying molecular defects in GT give rise to quantitative or qualitative defects in the GP IIb-IIIa complex. The lack of platelet aggregation to any concentration of ADP, collagen, arachidonic acid, or thrombin but normal agglutination by ristocetin is a characteristic of this disease. It has been demonstrated that a thrombasthenia-like state can result from naturally occurring antibodies binding to and inhibiting the function of the GP IIb-IIIa complex. Some of these antibodies have been linked to the presence of myeloma [19], Hodgkin's disease [20], or non-Hodgkin's lymphoma [14]. In other cases, anti-platelet antibodies against GP IIb-IIIa have appeared after blood transfusion in patients with Glanzmann's thrombasthenia [21,22]. These are generally considered to be isoantibodies although autoantibody activity against residual platelet-bound traces of GP IIIa has been reported [18,22].

We now report a woman with a severe form of AITP and where a thrombocytopenic state is associated with the presence of an autoantibody that inhibits GP IIb-IIIa function. What makes this case particularly unusual is that the occurrence of the antibody is also associated with a previously undescribed general perturbation in the balance of glycoproteins within the platelet membrane and a noticeable upregulation of GP Ib.

MATERIALS AND METHODS

Case Report

The patient (E.B.) is a 63-year-old woman who presented for the first time 6 years ago with a hemorrhagic syndrome consisting mainly of epistaxis and gum bleeding. Her platelet count was 5,000/μl. Bone marrow aspiration was performed and the presence of megakaryocytes confirmed. She was hospitalized and AITP was diagnosed. There was no previous history of blood or platelet transfusion. In the absence of bleeding, other blood cell counts were normal, as were plasmatic coagulation factors. She was treated with steroids (prednisone 1 mg/kg/day) without success. One year later, she underwent splenectomy, which only led to a short improvement in her platelet count and the bleeding tendency. Her platelet size is heterogeneous and some large platelets are present (mean platelet volume ≈14 μ^3; control range 6–8 μ^3). She was then treated periodically with IVIgG (400 mg/kg/day for 4 days) during 1 year. IVIgG therapy results in transient improvements of her platelet counts, typically to 40,000–60,000/μl. However, there was often a rapid and progressive decrease to pre-infusion levels. In March 1995, the patient was examined for the first time in our department, and she has since been hospitalized on two occasions for severe bleedings. We noted that bleeding was not always associated with the period of lowest platelet count. Informed consent was obtained for all studied performed on blood from the patient.

Serum and Plasma

Serum was obtained from blood taken directly into glass tubes and incubated for 4 h at 37°C. Samples were further heated for 30 min at 56°C to inactivate complement. On occasion, plasma was obtained from blood anticoagulated with 3.8% wt/vol sodium citrate (9 vol blood: 1 vol anticoagulant). Control sera or plasma were from adult hospital staff.

Preparation of Washed Platelets

Venous blood was obtained from the antecubital vein and anticoagulated at 6 parts of blood for 1 part of acid-citrate-dextrose NIH formula A (ACD-A). Platelet-rich plasma (PRP) was prepared by centrifugation at 120*g* for 10 min at room temperature and washed platelets obtained according to our standard procedures [23]. The platelets were resuspended in a modified Tyrode buffer, pH 7.4, containing 137 mM NaCl, 2 mM KCl, 0.3 mM NaH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose, 12 mM $NaHCO_3$, 5 mM Hepes, and 0.35% (wt/vol) bovine serum albumin (BSA). Controls were adult hospital staff.

Isolation and Purification of IgG

IgG were isolated from patient's plasma or that from a control donor on immobilized Protein-A (Pierce, Rockford, IL). Volumes (3 ml) of plasma were passed at a rate of 0.8 ml/min through a Protein-A column equilibrated with phosphate-buffered saline (PBS), pH 7.2. After washing with PBS until the optical density (OD) at 280 nm returned to baseline, the adsorbed IgG were eluted with 0.2 M glycine-HCl buffer, pH 2.8. The IgG fractions were brought to pH 7.2, dialyzed, and concentrated to the initial volume by negative pressure dialysis against PBS [22].

Platelet Aggregation

These studies were performed using a Platelet Aggregation Profiler Model PAP-4 (Bio/Data Corporation,

Paris, France). Volumes (500 μl) of citrated PRP from the patient or a control donor were placed in the aggregometer cuvette at 37°C. On occasion, the platelet count of the control donor was adjusted to give a comparable number to that in the patient's sample. This was achieved by diluting the PRP with platelet-poor plasma [24]. The following agonists were tested: 1–8 μM ADP (Sigma Chimie, St. Quentin Fallavier, France), 25 μg/ml collagen (Diagnostica Stago, Asnières, France), 0.5 mg/ml arachidonic acid, 0.5–1.5 mg/ml ristocetin (Diagnostica Stago), and 50 μM thrombin receptor activating peptide (TRAP-14 mer) (Neosystem, Strasbourg, France). We also examined the aggregation given by 2 μM ADP using a mixture of 400 μl PRP from a control subject and 100 μl PRP from the patient. Aggregation with 0.5 and 0.1 U/ml thrombin was performed using 400 μl of washed platelets prepared as described above and resuspended at 10^8/ml in the modified Tyrode buffer.

The inhibitory effect of the patient's IgG on the aggregation response was studied using gel-filtered control platelets (10^8/ml) prepared as described [24] and incubated with 100 μl of isolated IgG (final concentration = 100 or 500 μg/ml) prior to the addition of ADP, Trap-14-mer peptide, or thrombin as detailed. Results were compared to those obtained when platelets were incubated with the same concentration of control IgG.

Murine Monoclonal Antibodies (MoAbs) Used in This Study

Bx-1 (anti-GP Ib), XIIF9 (anti-GP IIIa), and VH10 (anti-P-selectin) were prepared by us. The characterization of these antibodies has been reported before [22,23]. P2 (anti-GP IIb-IIIa), SZ22 (anti-GP IIb), ALB-6 (anti-CD9), FA6-152 (anti-GP IV), Gi9 (anti-GP Ia-IIa), and SZ2 (anti-GP Ib-IX) were purchased as isolated IgG from Immunotech (Luminy, France), Y2/51 (anti-GP IIIa) was purchased as isolated IgG from Dako (Glostrup, Denmark) and FMC25 (anti-GP IX) from Eurobio (Les Ulis, France). AP2 (anti-GP IIb-IIIa) and AP6 (an IgM recognizing an anti-LIBS [''ligand-induced binding site''] on GP IIIa) were generously made available by Dr. Thomas Kunicki (Scripps Research Institute, La Jolla, CA) [25,26]. F26 (recognizing an anti-RIBS [''receptor-induced binding site''] on fibrinogen) was kindly provided by Professor Harvey Gralnick (NIH, Washington, DC) [27]. PAC-1 (an IgM recognizing activated but unoccupied GP IIb-IIIa complexes) was purchased from the University of Pennsylvania, Philadelphia [28]. Saturating concentrations of each antibody were established by flow cytometry using nonfixed platelet suspensions.

Flow Cytometric Analysis of Platelet Membrane Glycoproteins Before and After Activation

Studies on fixed platelets. Washed platelets were adjusted to 10^8/ml in modified Tyrode buffer. In some experiments, aliquots were activated by 0.5 U/ml thrombin for 5 min or 20 mM PMA for 10 min at 37°C. The platelets were fixed in 1% (wt/vol) paraformaldehyde (PFA) for 30 min at 37°C. The PFA-fixed platelets (activated or not) were washed twice in modified Tyrode buffer and then sequentially incubated with different MoAbs and fluorescein isothiocyanate (FITC)-labeled affinity purified $F(ab')_2$ fragments of sheep anti-mouse IgG (Silenius, Victoria, Australia) or dichlorotriazinylamino fluorescein (DTAF)-conjugated affinity purified $F(ab')_2$ fragments of donkey anti-mouse IgM (Jackson ImmunoResearch Labs, West Grove, PA) (for PAC-1 and AP6) and analyzed with a FACScan flow cytometer (Becton Dickinson, Le Pont de Claix, France) as previously described [23,24]. Fluorescence histograms were obtained for 10,000 cells. Histograms were composed from fluorescence data obtained using gain settings in the logarithmic mode. Antibody binding was expressed as the percentage of platelets positive for the antibody or as mean fluorescence intensity (MFI) expressed on a linear scale. The gate for activated platelets was set to include < 1% of the events seen when identical test samples were incubated without the murine MoAb [24].

Studies on unfixed platelets. These were performed using citrated PRP according to the procedures of Bihour et al. [24]. In brief, aliquots of PRP (10 μl) were added to polystyrene tubes containing 100 μl modified Tyrode's buffer and a predetermined saturating concentration of the selected MoAb (see figure legends). Platelet agonists were present in the tube as detailed [24]. Control tubes contained either (1) an equivalent volume of buffer and the MoAb but no agonist or (2) the agonist and equivalent amounts of an irrelevant mouse IgG or IgM. After 15 min at room temperature without agitation, saturating amounts of FITC-labelled $F(ab')_2$ fragments of anti-mouse IgG or (DTAF)-conjugated affinity purified $F(ab')_2$ fragments of donkey anti-mouse IgM were added as described above. After 15 min in the dark, samples were diluted with 750 μl of modified Tyrode's buffer and analyzed immediately in the flow cytometer.

Flow Cytometric Analysis of Platelet Surface IgG and IgM (PSIgG and PSIgM)

Volumes (10 μl) of washed platelets from the patient or control subjects at 2.5×10^8/ml were incubated for 30 min at room temperature with 100 μl of FITC-labeled anti-human IgG (γ-chain) $F(ab')_2$ or FITC-labeled anti-human IgM (μ-chain specific) (Jackson ImmunoResearch) diluted 1/1,000 in modified Tyrode buffer. The platelet suspension was then diluted with 750 μl modified Tyrode buffer prior to analysis using the FACScan as described elsewhere [29]. The mean value for PSIgG and PSIgM obtained for 20 healthy control donors was 8.2 ± 1.8 (PSIgG) and 7.9 ± 2 (PSIgM) arbitrary units.

Results for patients are considered positive when the MFI exceeds this mean by 3 SD.

Screening of Anti-Platelet Antibodies in Human Serum

Binding of immunoglobulins (IgG + IgM) to PFA-fixed washed platelets from normal individuals was assessed by an ELISA assay [30]. Characterization of the anti-platelet antibodies was performed by MAIPA [1]. Test platelets were resuspended in PBS, pH 7.4, containing 0.1% (wt/vol) sodium azide and stored overnight at 4°C prior to use. After centrifugation at 1,200*g* for 10 min, the platelets were resuspended at 2×10^9/ml in PBS, pH 7.2, containing 2% (wt/vol) BSA (PBS-BSA). Aliquots (20 μl) were incubated at room temperature following the addition of either 300 μl decomplemented serum from (E.B.) or purified IgG as described in Results. As a positive control, we used serum from a polytransfused patient with type I Glanzmann's thrombasthenia (E.B.V.) who possesses a high titre antibody to GP IIIa [22]. Volumes (15 μl) of a saturating concentration of one of the above-mentioned murine MoAbs (in most cases 5–10 μg/ml) were added simultaneously. The rest of the procedure was as previously described by us [29,31,32].

Screening of Platelet-Associated Antibodies

A quantitative assessment of total platelet IgG in Triton X-100-soluble lysates prepared from washed platelets of the patient was performed using an ELISA technique and microtitration plates coated with goat anti-human IgG antibody specific for Fc fragments (Jackson ImmunoResearch) as described before [29]. The specificity of IgG associated with the patient's own platelets was assessed using the same Triton X-100-soluble platelet lysates and our recently developed PAICA assay. Here, platelet antigens were captured by one of the above-mentioned MoAbs prebound to the wells of a microtitre plate and the presence of associated human immunoglobulin assessed using alkaline-phosphatase-conjugated affinity-purified $F(ab')_2$ fragments of a goat anti-human IgG (Jackson ImmunoResearch) [29].

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Platelet Proteins and Western Blotting

Washed platelet suspensions were prepared and solubilized for SDS-PAGE according to our previously described procedures [22]. Samples containing 50 μg SDS-soluble platelet extracts were analyzed by SDS-PAGE performed using 7 to 12% gradient acrylamide slab gels with a 3% stacking gel. On occasion, disulphide reduction was performed by heating at 100°C for 5 min in the presence of 100 mM dithiothreitol. Protein standards for molecular mass determinations were purchased from Bio-Rad (Ivry-sur-Seine, France). Proteins were detected by Coomassie blue R-250 staining. Proteins resolved following SDS-PAGE were transferred onto nitrocellulose sheets and processed as already described [22]. Serum or plasma were diluted 1:10 with PBS prior to use. MoAbs were used at the dilutions described in the flow cytometry experiments. They are identified in Results. On occasion, individual strips were incubated with a combination of Bx-1 + SZ22 + XIIF9 each being at 5 μg/ml; this allowed the simultaneous detection of GP Ib, GP IIb, and GP IIIa. Bound antibodies were revealed using horseradish peroxidase-conjugated anti-human IgG or anti-mouse IgG (Jackson ImmunoResearch) diluted 1/1,000 and a 6% (vol/vol) solution of 3 mg/ml 4-chloro-1-naphthol in methanol with 0.04% (vol/vol) H_2O_2 in 10 mM Tris-HCl, pH 7.4 [22].

RESULTS

Platelet Function Testing

Platelet aggregation was performed using the patient's citrated PRP during periods when her platelet count permitted. The platelet count of the control PRP was adjusted to that of the patient with PPP. Platelets from (E.B.) showed little or no response to ADP (1–8 μM), collagen (25 μg/ml), arachidonic acid (0.5 mg/ml), or TRAP-14 mer peptide (50 μM). In contrast, the platelets showed a normal response to ristocetin (1 and 1.5 mg/ml). Typical results from ADP, collagen and ristocetin are illustrated in Figure 1. Washed platelets from (E.B.) also showed a decreased response to thrombin (Fig. 1). Similar results were observed for platelets tested on three separate occasions since March 1995. We also tested the aggregation of gel-filtered normal platelets incubated for 30 min with IgG isolated from the patient's serum by chromatography on protein A-Sepharose. As shown in Figure 2, the patient's IgG at 500 μg/ml was a powerful inhibitor of platelet aggregation induced by ADP, TRAP-14 mer, and thrombin. Results are compared to those obtained following the addition of the equivalent amount of nonimmune IgG. Significant inhibition was also seen with 100 μg/ml IgG from (E.B.) serum (not shown).

Platelet-Surface IgG and IgM

PSIgG and PSIgM were assessed following the incubation of washed platelets from (E.B.) or a control donor with FITC-labeled $F(ab')_2$ of Fc-γ-chain specific goat anti-human IgG or Fc-μ-chain specific anti-human IgM. As shown in Figure 3A, the patient's platelets were highly positive for IgG, with virtually all of the platelets exhibiting bound antibody. The MFI ranged from 80 to 191 on the three occasions that surface IgG was tested, results that were highly significant. No increase of MFI was observed when platelets of the patient were incubated with FITC-labeled anti-human IgM (Fig. 3B). The

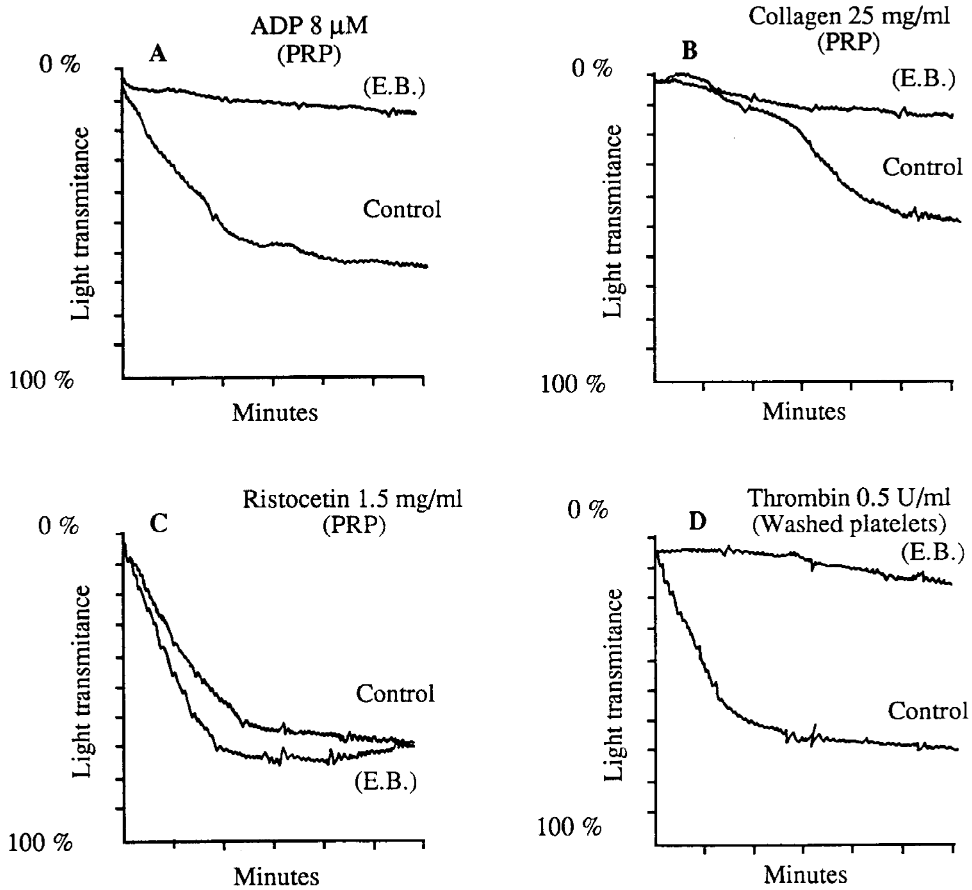

Fig. 1. Platelet aggregation responses obtained using citrated PRP (0.59 × 10^8 platelets/ml) from the patient (E.B.) or a control donor (0.8 × 10^8 platelets/ml). Illustrated are the results obtained using ADP (8 µM), collagen (25 µg/ml), and ristocetin (1.5 mg/ml). Only ristocetin gave a significant response. The aggregation of washed platelets with 0.5 U/ml thrombin was performed using (E.B.) and control platelets at 10^8 platelets/ml.

washed platelets showed no signs of α-granule secretion, P-selectin not being expressed (see ''Platelet Membrane Glycoprotein Expression''). In our laboratory, PSIgG and PSIgM are considered positive in flow cytometry when the MFI values exceed by 3 SD the average of the values obtained for 20 healthy control donors (MFI = 8.2 ± 5.4 for PSIgG and MFI = 7.9 ± 2 for PSIgM) [29].

Characterization of Antibodies to Platelet Glycoproteins in (E.B.) Serum

The presence of anti-platelet antibodies in the serum of the patient was first evaluated by adsorption to PFA-fixed control platelets, bound immunoglobulin being detected in ELISA using polyclonal antibodies reactive with both IgG and IgM. A ratio of 1.37 with respect to control serum represented a weakly positive response [see 29]. The specificity of the anti-platelet serum antibodies was tested in MAIPA performed using a panel of murine MoAbs directed against the major glycoproteins of the platelet membrane. As shown in Figure 4A, results with MoAbs against GP IIIa (XIIF9, Y2/51) were strongly positive yet they were negative with MoAbs specific for GP IIb (SZ22) and complex-dependent determinants on GP IIb-IIIa (AP2 and P2 [not shown]). This specificity was found using sera obtained at well-separated intervals with little changes noted in the antibody titre. Results were borderline with Bx-1 (anti-GP Ib), only reaching statistical significance on one occasion. Results were negative with Gi9 (anti-GP Ia-IIa), ALB-6 (anti-CD9), and FA6-152 (anti-GP IV) (not shown). The MAIPA assay was also performed using control platelets incubated with IgG isolated from the patient's serum. As a positive control, platelets were also incubated with the IgG from the serum of a polytransfused Glanzmann's thrombasthenia patient previously shown to contain an IgG antibody to GP IIIa [22]. Murine MoAbs used in this experiment were XIIF9 and AP2. An optical density in excess of 2 was obtained using XIIF9 (anti-GP IIIa) for both (E.B.) serum and that from the Glanzmann's thrombasthenia patient. In con-

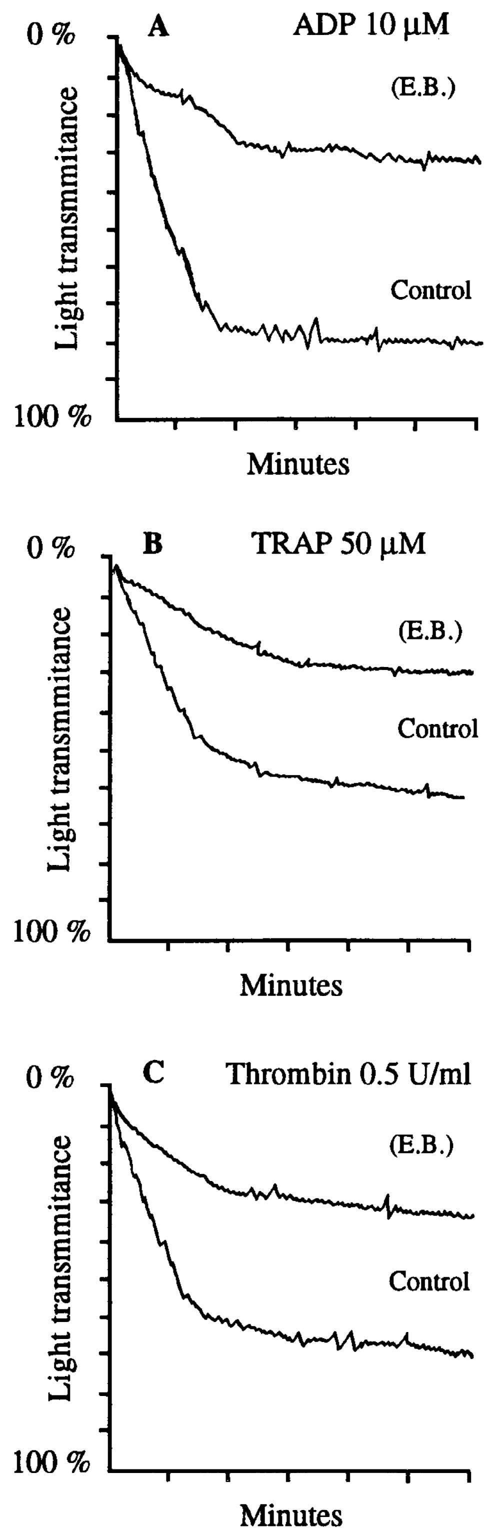

Fig. 2. Gel-filtered control platelets (10^8/ml) were incubated with the isolated IgG (500 μg/ml) from (E.B.) plasma or from that of a control donor for 30 min at 37°C. Platelets were then stimulated with ADP, TRAP-14-mer peptide, or thrombin at the indicated concentrations. The aggregation with ADP was performed in the additional presence of 200 μg/ml fibrinogen.

trast, results obtained using AP2 (anti-GP IIb-IIIa) were negative for (E.B.) but remained strongly positive for the serum from the Glanzmann's patient (data not shown).

Quantification and characterization of platelet IgG (PAIgG). In our laboratory, PAIgG are considered elevated in ELISA when the IgG concentration exceeds by 3 SD the mean of the values obtained for 20 healthy control donors (2.6 ± 0.53 fg/platelet) [29]. The total IgG content within the platelets of (E.B.) was 26 fg/platelet, testifying that in this patient the amount of platelet surface IgG and/or IgG within internal stores was considerably increased. Using the same lysate, we also determined whether platelet-associated IgG showed the same specificity as the antibodies detected in the patient's serum. For this characterization we used PAICA, a procedure that consists of first attaching to the ELISA wells a murine MoAb directed against a selected membrane glycoprotein and then of adding a volume of the platelet lysate containing the glycoprotein target. Human antibody associated with the target antigen is revealed colorimetrically. As shown in Figure 4B, results mirrored those obtained for the serum antibodies. It is therefore reasonable to assume that platelet-bound antibodies of (E.B.) and those in the serum had similar specificity for GP IIIa.

Platelet Membrane Glycoprotein Expression on (E.B.) Platelets Before and After Activation

Studies were performed using a series of MoAbs in flow cytometry. On resting platelets from (E.B.), AP2 (anti-GP IIb-IIIa) gave a symmetrical histogram (Fig. 5), although the MFI was weaker than that seen for platelets from a series of control donors studied in parallel (Table I). The results for MoAbs reacting with epitopes specific for GP IIb-IIIa (P2) or GP IIb (SZ22) paralleled those obtained with AP2. Results for CD9 were also low. In contrast, Bx-1 (anti-GP Ib) showed a dramatic increase in binding compared with that normally seen (Fig. 5). Other MoAbs specific for GP Ib (AP1), GP Ib-IX (SZ2), or GP IX (FMC-25) showed parallel increases, indicating that the entire GP Ib-IX complex was present in much greater amounts at the platelet surface (Table I).

We next studied the response of washed platelets from the patient to activation by thrombin. The thrombin-induced expression of P-selectin, a marker of α-granule secretion and revealed using VH10, was low on (E.B.) platelets. In fact, it appeared that only a subpopulation of the platelets were responding to the stimulation. The surface expression of P-selectin was more homogeneous when (E.B.) platelets were incubated with PMA, which bypasses surface receptors and directly activates protein kinase C [33]. Platelets from (E.B.) also showed a reduced surface clearance of GP Ib-IX in response to thrombin (Fig. 5). Finally, the platelet activation re-

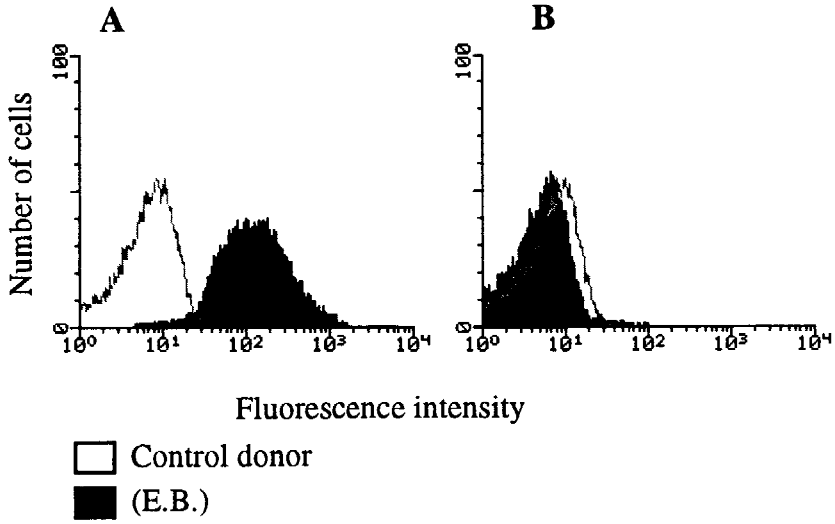

Fig. 3. Fluorescence histograms showing the detection of IgG (A) and IgM (B) on the surface of platelets from patient (E.B.) and a control donor as detected in flow cytometry. In the illustrated experiment, the MFI for IgG on (E.B.) platelets was 130.

sponse was studied using PAC-1, an anti-LIBS (AP6) and an anti-RIBS (F26) MoAb, which recognize 3 types of activation-dependent epitope on the GP IIb-IIIa complex. Platelets from the patient (E.B.) showed little binding of these three MoAbs when stimulated with a high dose of ADP (Fig. 6). Very similar results were obtained for platelets stimulated with thrombin and fixed prior to MoAb addition (F26 was not used here as its epitope is lost after PFA fixation) (Fig. 6).

SDS-PAGE and Western Blotting

Platelets from (E.B.) were analyzed by SDS-PAGE and the protein pattern revealed following CBR-250 staining. All major bands were located including those of the α-granule proteins fibrinogen and thrombospondin. The results of Western blotting performed using several MoAbs to the major membrane glycoproteins are shown in Figure 7. In lanes M are illustrated the results obtained using a mixture of MoAbs to GP Ib, GP IIb and IIIa, respectively, for samples from (E.B.) and a control donor. A decreased concentration of GP IIb and GP IIIa was visually apparent on the pattern obtained for (E.B.) platelets and this was confirmed by densitometric scanning (not shown). GP Ib was strongly present and a surprise was an additional band at ≈250 kDa on the (E.B.) platelet profile that was strongly recognized by all MoAbs to GP Ibα, but not by MoAbs to other membrane glycoproteins including FMC25 to GP IX. The migration and reactivity of this band with MoAbs was unchanged after disulfide reduction, suggesting that it contained GP Ibα-like domains but not GP Ibβ (not illustrated). An additional band previously calculated to be ≈176 kDa on the profile given by XIIF9 alone probably represents GP IIIa dimer and has been previously seen on the pattern given by control platelets [22].

DISCUSSION

The patient that we have described has a bleeding disorder in which a Glanzmann's thrombasthenia-like condition is associated with the presence of a moderate to severe thrombocytopenia and an autoantibody reacting with the GP IIb-IIIa complex. Platelet aggregation was defective with all agonists tested except for ristocetin. Unexpectedly, a study of the membrane glycoprotein expression in the patient's platelets by both flow cytometry and Western blotting showed a diminution in the platelet content of GP IIb-IIIa complexes whereas GP Ib-IX complexes were overexpressed. CD9 appeared also to have a decreased expression. The patient's clinical history shows that her hemorrhagic diathesis is probably acquired since excessive bleeding was not noted during childhood or during pregnancy and delivery. Her adult son is unaffected and has experienced none of the bleeding problems of his mother.

Recurrent episodes of severe bleeding after splenectomy occurred despite platelet counts at about 40,000/μl and led us to consider the presence of an anti-platelet antibody that was inhibiting platelet function. This was confirmed by the location of IgG antibodies to GP IIb-IIIa both in the patient's serum and on her platelets, and a thrombasthenia-like aggregation defect was demonstrated. Characterization of the serum antibodies using the MAIPA assay showed what was an unusual specificity in our experience. Strongly positive results were obtained when using XIIF9 or Y2/51, murine MoAbs to GP IIIa, yet results were negative with other MoAbs recognizing GP IIb or complex-dependent epitopes on GP IIb-IIIa. Results were also negative with all MoAbs to other membrane glycoproteins tested except for a threshold positivity on one occasion with Bx-1 (anti-GP Ib). A positive reaction with XIIF9 (anti-GP IIIa) but a negative one with AP2 (anti-GP IIb-IIIa) implies that the human antibody either (1) changes the conforma-

tion of GP IIb-IIIa so that AP2 cannot bind or (2) binds to an epitope close to or identical with that recognized by AP2 so that the first antibody to bind inhibits the binding of the second. The fact that both AP2 [25] and (E.B.) antibodies block platelet aggregation favours that they recognize closely associated determinants. It is interesting to compare these results with those obtained by us in a past study in which anti-platelet antibodies from two Glanzmann's thrombasthenia patients were characterized in MAIPA [22]. Here, both human antibodies reacted with GP IIIa in Western blotting and tested positively in MAIPA with a series of MoAbs to GP IIb-IIIa (including AP2), although one of them gave a much reduced response with AP3 (anti-GP IIIa). It would seem that (E.B.) antibody recognizes a different determinant that is close to a functionally important site on the complex. A survey of previous studies on AITP patients suggests that autoantibodies recognizing complex-dependent determinants on GP IIb-IIIa may not be uncommon in this disease [6,7,34].

Tests repeated on several occasions showed that platelets from (E.B.) possessed much higher than normal levels of both total and surface PAIgG. The relatively low reactivity of (E.B.) serum with PFA-fixed control platelets could suggest the destruction of the epitope recognized on GP IIb-IIIa during PFA-fixation. Using our recently developed PAICA test, which characterizes PAIgG in a whole platelet lysate much as the MAIPA test characterizes serum antibody [29], we found the same specificity against GP IIb-IIIa for platelet-associated antibodies as was seen for those in the serum. Thus, the antibody present in (E.B.) serum is a true autoantibody reacting with an extracellular domain of GP IIb-IIIa on the patient's own platelets. It should be emphasized that the patient has no history of blood transfusions before the onset of her hemorrhagic diathesis and, although she is a mother with an adult son, her antibody reacted with the platelets of all donors tested and independently of the HPA-1 phenotype (results not given). All of the data is compatible with the fact that this GP IIb-IIIa-reactive antibody has been formed through an autoimmune process.

Platelet function testing showed that (E.B.) has a severely decreased platelet aggregation in response to all physiologic agonists tested while the GP Ib-mediated response to ristocetin was normal, results that recalled those seen in Glanzmann's thrombasthenia [18]. The ability of IgG isolated from (E.B.) serum to inhibit aggregation of control platelets confirmed the relationship between the presence of the antibody and the lack of the platelet aggregation response in the patient. However, although platelet aggregation was deficient in (E.B.), the secretory response to thrombin was also reduced as evidenced by a reduced P-selectin expression. A lower than normal release reaction has been noted in Glanzmann's thrombasthenia [35], perhaps because the close platelet-to-platelet contact normally associated with platelet aggregation amplifies signal transduction. Alternatively, the anti-GP IIb-IIIa antibody could itself down-regulate platelet function. Transmembrane signalling has been shown to occur as a consequence of ligand binding to GP IIb-IIIa [36,37].

Although there are several reports of autoantibodies to GP IIb-IIIa that induce a thrombasthenia-like state [reviewed in 38], patient (E.B.) is one of the few examples where the presence of an inhibitory antibody is also associated with a severe thrombocytopenia. Furthermore, (E.B.) is unique in that her platelets also possessed an atypical membrane glycoprotein profile. This first came to light when her platelets were incubated with a series of MoAbs direct against different glycoproteins and their

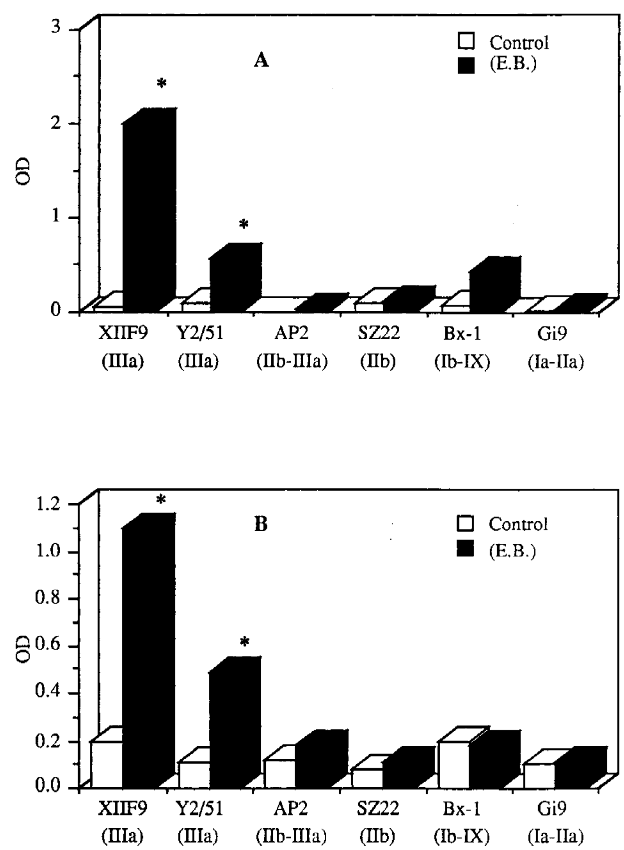

Fig. 4. Characterization of the specificity of the antibodies located in the serum of (E.B.) and on her platelets. A: Results obtained using a series of MoAbs to membrane glycoproteins in a standard MAIPA assay in which control platelets were co-incubated with an individual MoAb and either (E.B.) serum or that from a control donor with a normal platelet count. B: Results of the PAICA assay performed using whole platelet lysates from (E.B.) or a control donor and the same MoAbs. Tests were performed in duplicate. The control studies for each Mab are the mean optical density (OD) values obtained using platelet lysates from 3 normal donors.

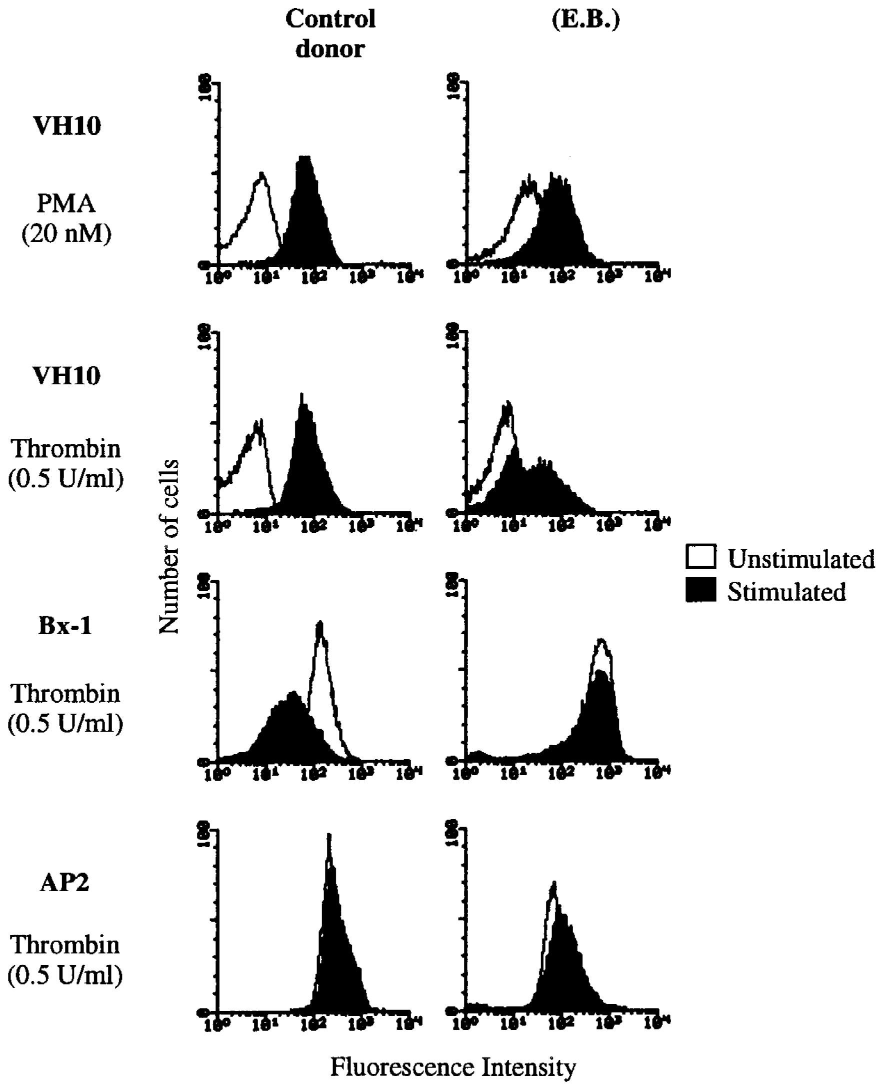

Fig. 5. Fluorescence histograms obtained when (E.B.) platelets or those from a control donor were incubated with selected monoclonal antibodies to platelet glycoproteins. Studies were performed on washed resting platelets or those stimulated with 0.5 U/ml thrombin. Platelet suspensions (10^8/ml) were fixed with PFA prior to being incubated for 30 min with the chosen MoAbs at the following concentrations: AP2 (anti-GP IIb-IIIa; 5 μg/ml), Bx-1 (anti-GP Ib; 5 μg/ml), and VH10 (anti-P-selectin; 5 μg/ml). Bound murine IgG was detected using FITC-labeled anti-IgG antibody.

binding was analyzed by flow cytometry. A diminution of the MFI compared with normal platelets was noted for murine MoAbs directed against GP IIb-IIIa. Although this may be related to the presence of human IgG already attached to the complex, Western blotting also revealed lower than normal amounts of GP IIb-IIIa. The observation that CD 9 antigen levels were also decreased but that murine MoAbs directed against different epitopes within the GP Ib-IX complex (GP Ibα or GP IX) bound in much higher amounts than to the control platelets, suggests a more generalized perturbation in membrane glycoprotein levels. It is difficult to explain the abnormal membrane glycoprotein profile. The fact that (E.B.) possesses occasional large platelets may have some influence. However, in giant platelet syndromes, the general tendency is that glycoproteins unaffected by genetic defects are present in increased numbers per platelet. However, in the case of (E.B.), GP Ib-IX complexes show a large increase while GP IIb-IIIa levels decrease, showing an altered glycoprotein balance.

TABLE I. Membrane Glycoprotein Expression on (E.B.) Platelets*

MoAbs	Control donor	(E.B.)
AP2 (anti-IIb-IIIa)	398	142
P2 (anti-IIb-IIIa)	362	129
SZ22 (anti-IIb)	249	70
ALB6 (anti-CD9)	310	94
Bx-1 (anti-Ib)	205	560
AP1 (anti-Ib)	223	659
SZ2 (anti-Ib-IX)	196	590
FMC25 (anti-GP IX)	111	422

*Studies were performed on PFA-fixed platelets by flow cytometry, with data expressed as mean fluorescence intensity (MFI). Results are given for the control donor and (E.B.) tested in parallel. Comparative studies for a series of 10 control donors in our laboratory are 390.3 ± 27.7 (AP2), 183.2 ± 19.33 (Bx-1), and 105.6 ± 21.3 (FMC-25) (mean ± SD).

A major feature is that Western blotting experiments showed the presence of a 250 kDa band that reacted with a series of MoAbs to GP Ibα. The nature of this band is unknown but its unchanged migration after disulfide re-

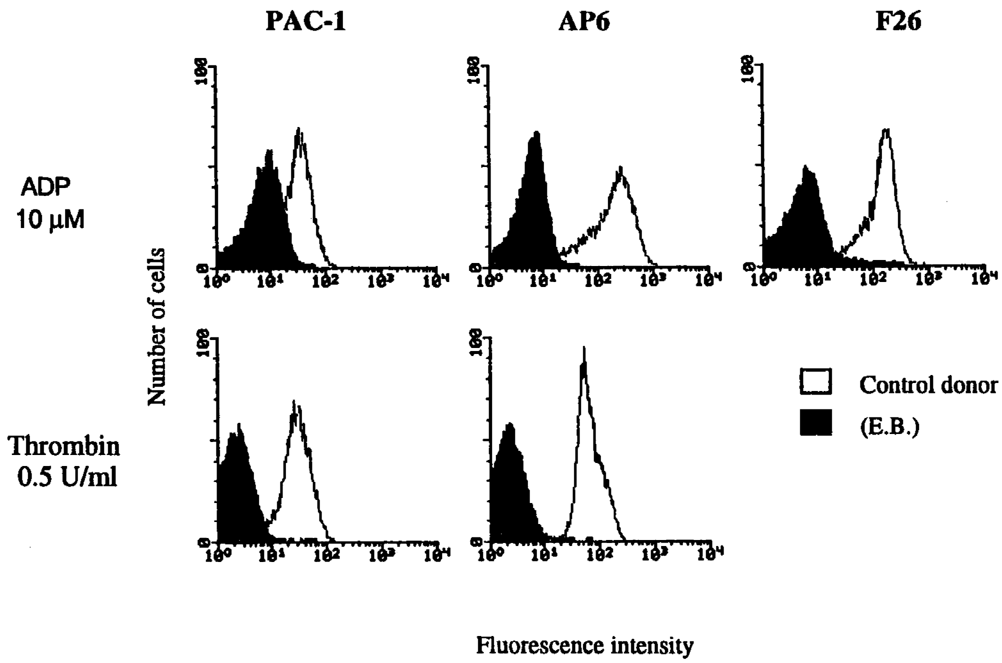

Fig. 6. Fluorescence histograms showing the binding of MoAbs that recognize different categories of activation-dependent antibodies on GP IIb-IIIa complexes. Citrated PRP from (E.B.) or a control donor was incubated at 37°C in the presence or absence of 10 µM ADP in the presence of PAC-1 (5 µg/ml), F26 (5 µg/ml), or AP6 (5 µg/ml). Experiments with thrombin were performed using washed platelets as described in the legend to Figure 5 and here platelets were fixed with PFA prior to antibody addition. Bound mouse immunoglobulin was detected using FITC-labeled anti-mouse antibody selective for IgG or IgM (for PAC-1 and AP6). Only results for activated platelets are shown.

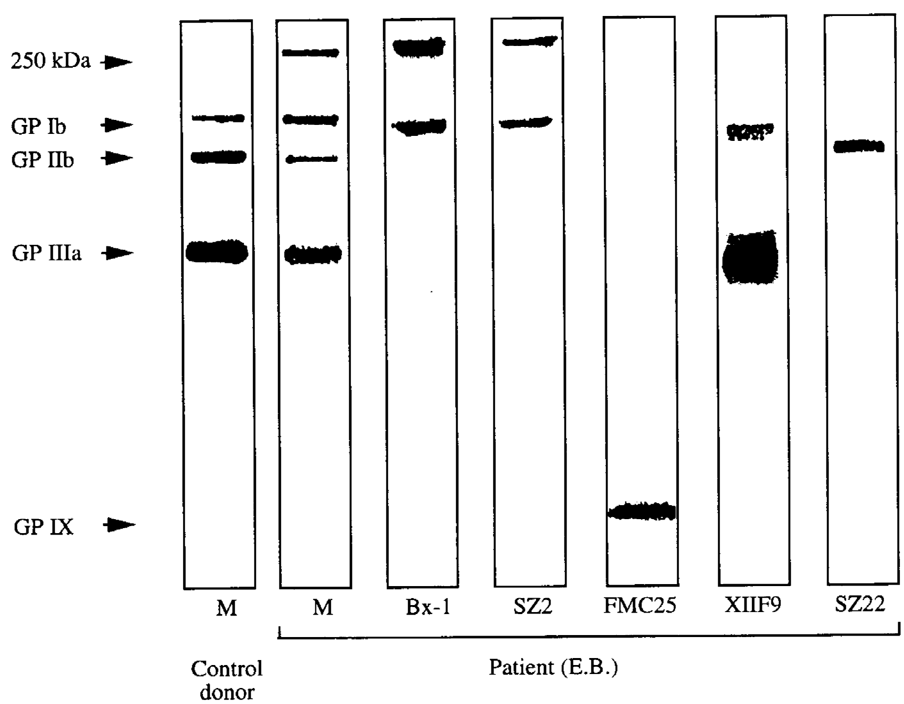

Fig. 7. Western blotting experiments showing the presence of the major membrane glycoproteins in (E.B.) platelets. SDS-soluble extracts (50 µg protein) of platelets from (E.B.) and a control donor were subjected to SDS-PAGE on 7–12% gradient gels prior to being transferred to nitrocellulose membrane. Incubation with MoAbs was performed either as a mixture (M) (Bx-1, XIIF9, and SZ22) or individually. Bound murine IgG was revealed using peroxidase-labeled second antibody.

duction suggested that the normally disulphide-linked GP Ibβ was not present. Southern blotting experiments with cDNA probes for GP Ibα and digested genomic DNA from (E.B.) have failed to reveal any abnormality (de la Salle and Lanza, unpublished data). One possibility is that GP Ibα is synthesized in excess and is present in an SDS-resistant complex in the platelets of (E.B.). The fact that the patient has a severe hemorrhagic diath-

esis and few platelets precludes the structural characterization of this band or the isolation of mRNA for GP Ibα from her platelets. High mol wt bands, which represent ''GP Ib-like'' proteins, have been described [39,40], although the relationship between the band seen after Western blotting of (E.B.) platelet proteins and those described in previous publications remains to be determined. It is interesting that ristocetin-induced platelet agglutination was normal, showing that the GP Ib was able to bind von Willebrand factor. Yet, the platelet response to both ADP and thrombin was poor and this extended to both P-selectin expression and the expression of the activation-dependent epitopes on GP IIb-IIIa necessary for fibrinogen binding. A defect in signalling is, therefore, to be anticipated in addition to the direct inhibitory effect of the autoantibody on platelet aggregation through GP IIb-IIIa blockade.

Is there a relationship between the altered membrane glycoprotein composition and the presence of the autoantibody to GP IIb-IIIa? Although many studies have shown the presence of antibodies to GP IIb-IIIa in patients with chronic AITP (see Introduction), antibody characterization is not generally accompanied by an exploration of the membrane glycoprotein composition of the patient's platelets. Thus the extent to which (E.B.) represents a unique case is unknown. As GP IIb-IIIa complexes are present in precursor cells early in megakaryocytopoiesis [see 41], we feel that the possible effect of autoantibodies on megakaryocyte development and the level of synthesis of membrane glycoproteins would be worthy of study.

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